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The Sugar Spectrum of Human Cholesterol Gallstones, Mixed and Pigment Gallstones: Combined Quantitative Analysis of Neutral Sugars, N-Acetylhexosamines, Hexuronic and N-Acetylneuraminic Acids by Capillary Gas-Liquid Chromatography

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Summary: Glycoproteins were investigated in different types of biliary calculi by a methanolysis procedure of the carbohydrate moiety, the formation of 2,6-dinitro-4-trifluoromethylbenzenesulphonate derivatives of N-acetylhexosamines and N-acetylneuraminic acid, followed by separation, identification and quantification of the liberated monosaccharides using capillary gas-liquid chromatography.

This indirect method avoids several analytical problems caused by the presence of bile pigment derivatives; unless these are carefully removed they interfere strongly in the measurement of protein. The carbohydrate contents were much higher in pigment stones than in cholesterol and mixed stones.

Rhamnose, a non-mammalian monosaccharide, was identified in most samples, but was significant increased in pigment stones, indicating a bacterial history of this stone type.

Das Zuckerspektrum von Cholesterin-Gallensteinen, gemischten Gallensteinen und erdigen Pigment-Gallensteinen des Menschen:
Kombinierte quantitative Analyse der Neutralzucker, N-Acetylhexosamine, Hexuronsäuren und der N-Acetylneuraminsäure mit Hilfe der Kapillargaschromatographie

Zusammenfassung: Der Zuckeranteil der Glykoproteine verschiedener Gallensteintypen wurde nach Methanolyse in Form der O-Glykoside der Monosaccharide erfaßt. Die N-Acetylhexosamine und die N-Acetylneuraminsäure wurden in ihre 2,6-Dinitro-4-trifluoromethylbenzylsulfonsäurederivate überführt. Die Trennung, Identifizierung und Mengenbemessung der freigesetzten Zucker wurde auf kapillargaschromatographischem Wege erreicht.

Mit dieser indirekten Methode des Glykoproteinnachweises konnten verschiedene Schwierigkeiten umgangen werden, die mit der Proteinbestimmung verknüpft waren; denn die naßchemische Bestimmung von Proteinen in Gallensteinen wird erheblich durch Gallenfarbstoffe gestört, wenn diese nicht restlos entfernt werden. Wir stellten fest, daß sich Pigmentsteine und Cholesterinsteine sowie gemischte Steine in ihrem Zuckergehalt eindeutig voneinander unterschieden, d.h. der Kohlenhydratanteil der erdigen Pigmentsteine war wesentlich höher als jener der gemischten Steine und der Cholesterinsteine.

In den meisten Steinen wurde Rhamnose gefunden, ein Zucker, der nicht im Stoffwechsel der Säugetiere vorkommt. Auffällig war sein signifikant erhöhter Anteil in Pigmentsteinen, was auf eine bakterielle Infektion bei deren Entstehung hindeutet.

Introduction

Since in 1896, when *Naunyn* (1) suggested that bile proteins might be involved in gallstone formation, this subject has been the subject of vigorous discussion (2–15). First of all the composition of proteins, glycoproteins, and mucous material from human bile and from the biles of animals fed a lithogenic diet was studied by several investigators (3, 6, 9, 12, 13, 15, 16). Analysis of the mucous material in gallstones is subject to considerable interference by bile pigments present in biliary calculi (15, 16). Nevertheless several authors have reported analyses of the proteins and glycoproteins in stones without paying attention to this interference or the additional interference by some solvents or dyes used in chemical detection and histochemical staining (8, 10, 14).

Another approach to the investigation of mucous substances in gallstones is the cleavage of the polysaccharide moiety into monosaccharides followed by identification and quantification using gas-liquid chromatography (GLC). This method should be subject to minimal interference by bile pigments and other gallstone ingredients. Unfortunately most of the preparation methods published so far are very complicated and time consuming, or, especially when acid hydrolysis is involved, they result in extended losses (15, 17, 19, 21). When packed columns were employed for sugar determination, retention times ranged from 30 min to 2 h (13, 17, 20, 21), resulting in broadening peaks, substance loss and deficient resolution. This may lead to errors in the classification and quantification of peaks when the mixture to be chromatographed is complicated, as is usually the case in gallstone extracts.

We have attempted to overcome such problems by improving the sugar assay in gallstones. Based on *Preuß & Thier* (22) we developed a procedure including methanolysis, derivatization with 2,6-dinitro-4-trifluoromethylbenzene sulphonate (DNTS), trimethylsilylation. and capillary GLC.

Materials and Methods

Gallstone material

The stones were from patients who underwent either cholecystectomy or endoscopic sphincterotomy, or stones were obtained at autopsy. We therefore had gallbladder and common bile duct stones (cholesterol line n= 36; pigment line[1]) n= 16; mixed line[2]) n= 17).

[1]) "Earthy" or calcium bilirubinate pigment stones.
[2]) Mixture of cholesterol and calcium bilirubinate.

Reagents

Methanol was purified and stored on molecular sieve 4 A° (Merck). Pyridine, analytical grade (Baker) was dried with KOH. Methanolic HCl and the reference solutions were prepared and standardized as described by *Preuß & Thier* (22). 2,6-Dinitro-4-trifluoromethylbenzene sulphonate (DNTS) was prepared according to *Gerig & Reinheimer* (23). Silylation was performed with hexamethyldisilazane (HMDS) from Sigma and with trimethylchlorsilane (TMCS) from Merck. 1,1,2-Trichlortrifluorethane (TCFE) from Merck was added to prevent the detector tip from obstruction by SiO_2.

Apparatus

GLC

Capillary GLC was performed with a Carlo Erba instrument ("Fractovap 2350"), equipped with a hydrogen flame ionization detector and combined with a plotter/printer integrator ("SP 4270", Spectra Physics). A Durabond DB-5 column (15 m × 0,331 mm I.D., J & W Scientific) was used with a temperature programme starting at 140 °C. The temperature was elevated at a rate of 4 °C/min up to 150 °C and then raised stepwise (8 °C/min) up to 300 °C. The flow rate of the carrier gas (nitrogen) was regulated by a 20:1 splitter at 1.4 ml/min. The injector temperature was 275 °C.

GLC/mass spectrometry (MS)

The GLC/MS measurements were performed with a Hewlett Packard instrument ("HP 5992 B"). Capillary column: 25 m × 0.33 mm I.D. Stationary phase: SE 52 (0.2 µm). Split: Open linkage restriction capillary.

Preparation of stone material

Gallstones were ground to a fine powder with an agate mortar or with an agate ball mill. The stone powder was desiccated under vacuum over P_2O_5. 15–1000 mg of stone powder were transferred to a screw-capped 15 ml Sovirel glass tube with a teflon seal. Samples below 20 mg were derivated directly. Samples of more than 20 mg were suspended in an appropriate volume of cold $CHCl_3/CH_3OH$ (2 + 1 by volume), acidified with anhydrous HCl (to 1 mol/l) in order to remove lipids and most of the pigments. The tubes were shaken gently overnight at 4 °C. After centrifugation at 4000 min^{-1} (Heraeus Christ Labofuge I) for 10 min the supernatant was carefully removed. The pellet was washed twice with petrol ether (b.p. 60–80 °C), dried and subjected to methanolysis (see below). In order to check for possible loss of monosaccharides by prematured methanolysis during lipid and pigment removal, a series of supernatants was evaporated to dryness and treated like the pellets.

Methanolysis

1 ml of 2 mol/l methanolic HCl was added to the pellet and the tube was firmly sealed. Methanolysis was performed at 100 °C for 4 h. After cooling the remaining HCl was carefully neutralized with 0.5 mol/l sodium methanolate. For the purpose of comparison defined amounts of starch and ovalbumin were submitted to the same procedure.

DNT-derivatives

1 ml of a methanolic DNTS solution (0.14 mol/l) was added. The beginning of the reaction was characterized by a slight yellow colouring which, however, was hardly recognizable in bile pigment rich samples. The tube was tightened and kept at 25–30 °C for 20 h. Methanolic solutions of internal standards were added and all solvent was carefully removed at 35 °C in a stream of nitrogen.

Silylation

The residue was redissolved in 500 µl of dry pyridine. 400 µl of HMDS and 200 µl of TMCS were added and the tube was tightly sealed. The mixture was shaken vigorously in order to suspend all solids and allowed to stand for 1 h at 60 °C. After cooling 200 µl of TCFE were added, the mixture was centrifuged, and 1–1.5 µl the supernatant were injected (GLC).

Standards

0.5–1 mg of all sugars (see below) which have been reported to be present in serum and intestinal glycoprotein were prepared as described above in the sections "Methanolysis, DNT-derivatives and Silylation". Reproducibility was checked by at least triplicate GLC runs. Individual response factors were calculated from the ratio $I_{An} \times F \times I_{is}^{-1}$; where I_{An} is the sum of the measured peak integrals of all anomers, I_{is} is the peak integral of the internal standard (sorbitol), and F is a correction factor for different weight volumes.

Rhamnose was additionally identified by GLC/MS, as described previously.

Reference substances

D(+)Xylose, *D*-glucuronic acid, *D*(+)galactosamine-HCl, *D*(+)glucosamine-HCl, α-*L*(−)fucose, *L*(+)arabinose, *D*-sorbitol and perseitol (internal standards), α-*L*-rhamnose, N-acetyl-*D*-galactosamine, N-acetyl-*D*-glucosamine, *D*(+)galactose, ovalbumin (Sigma); N-acetylneuraminic acid (Serva, Heidelberg); *D*(+)mannose, *D*(−)glucose, saccharose (Merck, Darmstadt); starch (commercial quality).

Results

Methanolysis and derivatization procedure

Neutral sugars and glucuronic acid behaved as reported by *Preuß & Thier* (22), even though they were treated by the modified procedure which allowed the detection of amino sugars simultaneously. The formation of isomeric methyl glycosides in defined ratios due to methanolysis allowed an undoubted identification of the individual sugars even in complicated chromatograms. The response factors (± 2.7 to ± 8% S.D.) were similar to those found by the above authors, and total linearity was given within the measured range (1000–40 µg).

Glucosamine, galactosamine, N-acetylglucosamine, N-acetylgalactosamine, and N-acetylneuraminic acid were detected and quantified after conversion into the DNT-derivatives which were stable for at least several weeks. Their response factors were much higher than those of neutral sugars; nevertheless the detectable amounts were in the range of nmoles, and the variation was less than 10%, except for N-acetylneuraminic acid (12%). The ratio of the anomeric peak areas of the N-acetylamino sugars showed some fluctuation, probably due to competing reaction velocities at the O-glycosidic C_1-linkage or the N-CO-linkage in C_2 of the sugar during methanolysis. In chromatograms where the peaks of N-acetylamino sugars are partially overlapped by others, account should taken of this when quantification is based on the remaining pure peaks (see fig. 1).

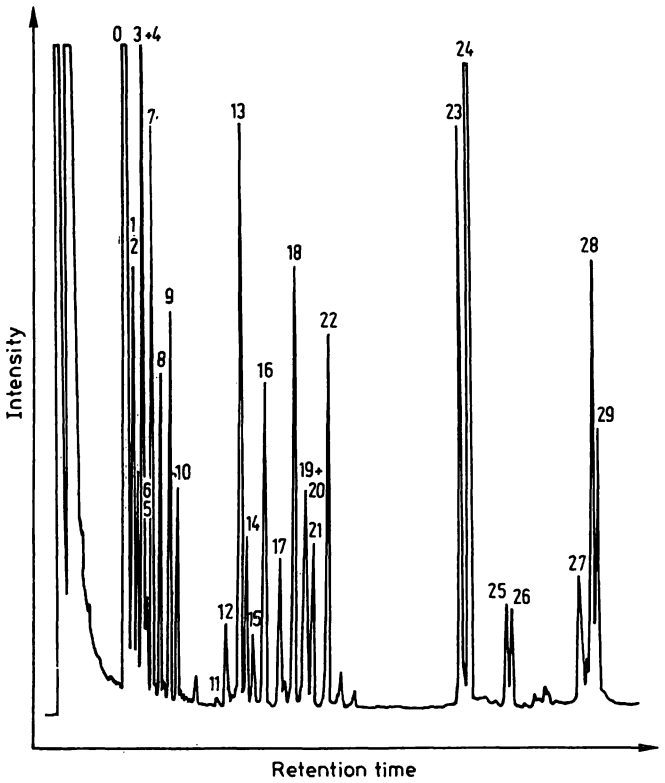

Fig. 1. Survey of the carbohydrates which have been found in intestinal glycoproteins (for derivatization and conditions of gas chromatographic separation see above).
Trimethylsilyl derivatives of
DNTS: 0;
arabinose: 1,2,6;
rhamnose:3,5;
fucose: 4,7,8;
xylose: 9,10;
glucuronic acid: 11,12,20,21;
mannose: 13,15;
galactose: 14,16,17;
glucose: 18,19;
sorbitol (internal standard): 22;
N-acetylneuraminic acid: 23,25,26;
perseitol (internal standard): 24;
N-acetylgalactosamine: 27,29;
N-acetylglucosamine: 28.

Methanolysis of starch appeared to be almost quantitative and the glucose recovery was found to be 99–99.5%, showing a good reproducibility (± 0.7% S.D.). Methanolysis of ovalbumin was also quantitative, but the reproducibility of the sugar analyses was somewhat inferior (mannose: ± 6% S.D., N-acetylglucosamine: ± 15% S.D.).

Partial resistance to methanolysis, as shown by polysaccharides of the hyaluronic acid and chondroitin sulphate type, was not observed in the carbohydrate moiety recovered from gallstones, i.e. pellet material (see above) undergoing methanolysis proved to be sugar-free when resubjected to methanolysis using a freshly prepared reagent. Sugar losses which might occur during extractive lipid removal were found to be negligible.

Cholesterol stones

From 36 cholesterol stone cases examined for neutral sugars and uronic acids 15 showed no traces of sugars. In 21 cases sugars were detected and in 18 of them the amounts were quantifiable: The range of the sugar content was 5–8455 μg/g, the mean 906 μg/g. Sugars were identified and measured as follows, with the number of cases shown in parentheses: arabinose (2), rhamnose (18), fucose (11), xylose (8), glucuronic acid (8), mannose (14), galactose (12), glucose (15).

The DNTS method had hitherto not been developed as a suitable assay for hexosamines and N-acetylneuraminic acid. From the very characteristic pattern of peaks resulting from decomposition during GLC, we concluded that five samples contained hexosamines (see tab. 1).

Mixed stones

Seven of 17 mixed stones contained carbohydrates (range: 180–2255 μg/g). The neutral sugar and uronic acid composition in these cases (mean sugar

Tab. 1. Sugars determined in cholesterol stones.

Case No.	Individual sugars (μg/g of stone powder) Arabinose	Rhamnose	Fucose	Xylose	Glucuronic acid	Mannose	Galactose	Glucose	N-Acetylneuraminic acid[1])	N-Acetylgalactosamine[1])	N-Acetylglucosamine[1])	Total
1	=	=	=	=	=	=	=	=				0
2	=	=	=	=	=	=	=	=				0
3	=	=	=	=	=	=	=	=				0
4	=	tr	214	tr	tr	tr	=	=				234
5	=	=	=	=	=	=	=	=				0
6	=	=	=	=	=	=	=	=				0
7	=	=	=	=	=	=	=	=				0
8	=	=	=	=	=	=	=	=				0
9	=	tr	tr	=	=	=	=	=				10
10	=	70	=	=	=	=	tr	80				155
11	tr	=	=	=	=	=	=	=				5
12	=	70	tr	tr	tr	tr	tr	80				175
13	=	=	=	=	=	=	=	=				0
14	=	10	16	tr	12	30	43	90				206
15	=	=	=	tr	tr	=	=	100				110
16	=	=	=	=	=	=	=	=				0
17	=	=	=	=	=	tr	tr	tr				15
18	=	150	tr	=	=	tr	60	100				320
19	=	=	=	=	=	=	=	=				0
20	=	tr	10	tr	20	30	40	80				190
21	=	250	tr	=	=	130	90	270				745
22	tr	100	40	40	170	50	=	190				595
23	=	=	=	=	=	=	=	=				0
24	=	70	=	=	tr	tr	tr	tr				90
25	=	=	=	=	=	=	=	=				0
26	=	=	=	=	=	=	=	=				0
27	=	=	=	=	=	=	=	=				0
28	=	=	=	=	=	=	=	=				0
29	=	250	=	=	=	=	=	=				250
30	=	700	tr	=	=	10	290	750				1755
31	=	226	74	16	=	tr	280	=				601
32	=	360	=	=	=	=	=	290				650
33	=	500	=	=	=	tr	tr	tr				515
34	=	2000	=	=	=	=	=	1000				3000
35	=	520	=	330	tr	1230	1070	5300				8455
36	=	930	tr	=	=	tr	=	=				940

= beyond the detection limit.
tr set to 5 μg/g.
[1]) not determined.

content 1152 μg/g) was similar to that of cholesterol stones. Ten samples showed no sugar traces. The remaing 7 stones had the following sugar pattern: rhamnose (7), fucose (6), glucuronic acid (3), mannose (6), galactose (6), glucose (7), N-acetylgalactosamine (3), N-acetylglucosamine (6) (see tab. 2, fig. 2).

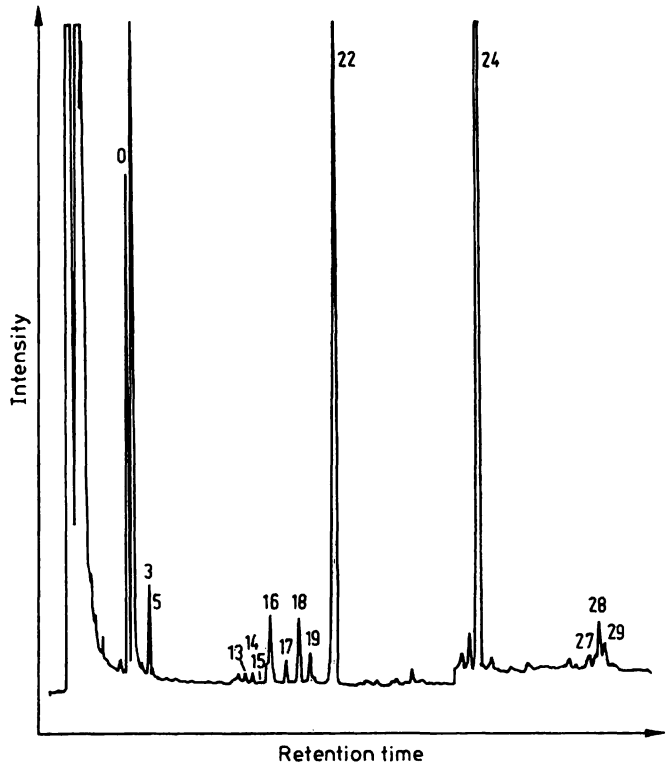

Fig. 2. Identification of carbohydrates: mixed stone type. Peak numbers as given in figure 1.

Pigment stones

All the 16 pigment stone samples examined contained several sugars as follows: rhamnose (16), fucose (8), xylose (4), glucuronic acid (9), mannose (13), galactose (12), glucose (14), N-acetylgalactosamine (5), and N-acetylglucosamine (10). The average sugar content was 2909 μg/g (range 15–11760 μg/g), related to initial dry weight (see tab. 3, fig. 3).

N-Acetylneuraminic acid could not be detected in any DNTS-derivatized samples.

Ratios of individual sugars

The sum of mannose, galactose, and glucose accounted for about 40–60% of the total sugars, glucose representing the major fraction. In cholesterol stones and mixed stones, fucose, xylose and glucuronic acid did not exceed 2% each, whereas in pigment stones glucuronic acid represented 14% of the total sugars. N-acetylgalactosamine and N-acetylglucosamine were of less significance in pigment stones (4%), but made up 23% of the total carbohydrate in mixed stones. Rhamnose was found to be a further main component in the sugar spectrum of gallstones, ranging from about 13% in mixed stones up to 35% in pigment stones (both figures related to total carbohydrates).

Tab. 2. Sugars determined in mixed stones.

Case No.	Individual sugars (μg/g of stone powder) Arabinose	Rhamnose	Fucose	Xylose	Glucuronic acid	Mannose	Galactose	Glucose	N-Acetylneuraminic acid	N-Acetylgalactosamine	N-Acetylglucosamine	Total
1	=	=	=	=	=	=	=	=	=	=	=	0
2	=	=	=	=	=	=	=	=	=	=	=	0
3	=	=	=	=	=	=	=	=	=	=	=	0
4	=	=	=	=	=	=	=	=	=	=	=	0
5	=	=	=	=	=	=	=	=	=	=	=	0
6	=	=	=	=	=	=	=	=	=	=	=	0
7	=	=	=	=	=	=	=	=	=	=	=	0
8	=	90	=	=	=	450	180	720	=	=	=	1440
9	=	tr	tr	=	=	90	=	70	=	tr	tr	180
10	=	140	tr	=	150	150	160	200	=	=	530	1335
11	=	tr	tr	=	=	70	140	350	=	110	80	760
12	=	=	=	=	=	=	=	=	=	=	=	0
13	=	tr	tr	=	tr	120	43	70	=	=	48	296
14	=	410	tr	=	tr	=	520	470	=	=	390	1800
15	=	=	=	=	=	=	=	=	=	=	=	0
16	=	=	=	=	=	=	=	=	=	=	=	0
17	=	500	tr	=	=	130	270	690	=	420	240	2255

= beyond the detection limit.
tr set to 5 μg/g.

Tab. 3. Sugars determined in pigment stones.

Case No.	Individual sugars (µg/g of stone powder) Arabinose	Rhamnose	Fucose	Xylose	Glucuronic acid	Mannose	Galactose	Glucose	N-Acetylneuraminic acid	N-Acetylgalactosamine	N-Acetylglucosamine	Total
1	=	40	110	tr	=	300	200	500	=	=	100	1255
2	=	200	320	=	570	1120	650	1650	=	tr	80	4595
3	=	tr	=	=	=	tr	=	=	=	=	tr	15
4	=	1000	tr	=	=	350	660	1400	=	=	120	3535
5	=	5200	=	=	3100	=	=	tr	=	tr	tr	8315
7	=	100	tr	=	=	450	310	1000	=	=	=	1865
8	=	tr	=	=	=	250	tr	980	=	920	220	2380
9	=	800	tr	=	=	tr	tr	840	=	=	=	1655
10	=	380	tr	tr	8	20	95	380	=	tr	78	976
11	=	230	72	tr	tr	100	95	410	=	=	94	1011
12	=	2200	84	=	630	62	1160	1270	=	220	150	5776
13	=	3600	=	=	2200	810	2620	2530	=	=	=	11760
14	=	540	=	=	tr	tr	tr	tr	=	=	=	560
15	=	850	=	=	tr	tr	tr	tr	=	=	=	870
16	=	120	=	920	=	=	=	940	=	=	=	1980

= beyond the detection limit.
tr set to 5 µg/g.

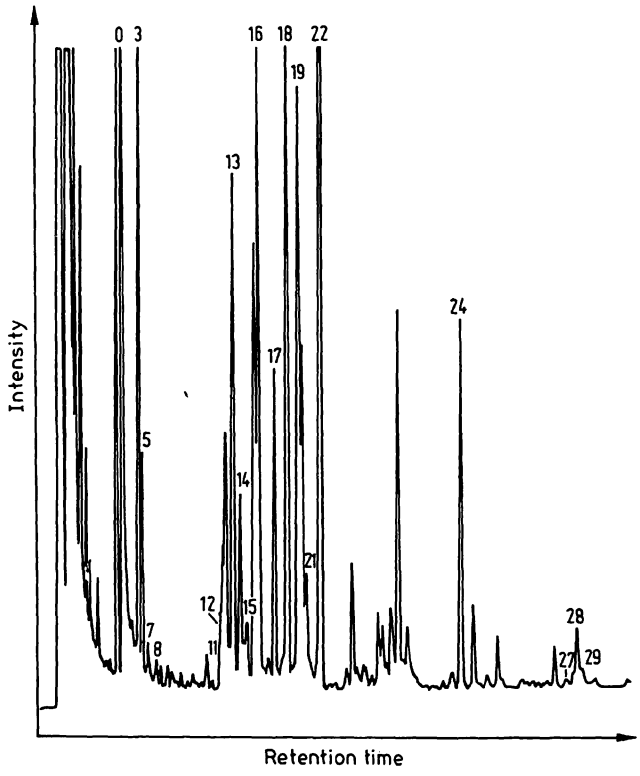

Fig. 3. Identification of carbohydrates: pigment stone type. Peak numbers as given in figure 1.

Discussion

In the analysis of glycoproteins in gallstone material with different common methods, i.e. *Lowry*'s protein test (24), coomassie protein test (25), amino acid assay with ninhydrin, and photometric protein determination at 280 nm, strong interference by pyrrolic compounds, bile salts, and solvents with nitrogen-derived functional groups (EDTA, TRIS, Triton X-100, guanidine etc.) has to be taken into consideration. *Pearson* et al. described a very strong interference by bile pigments in glycoprotein assays by the PAS-method (16). *Good* et. al. (10) who reported a mean value of 30% for protein in non-carbonate pigment stones used coomassie blue absorption at 595 nm and *Lowry*'s reagent for protein determination. The solvent was Na_2EDTA, and a satisfactory removal of pigment derivatives and bile salts was not performed. *Matsushiro & Nemoto* (26) found 2.5–2.8% of sugar compounds in acetone-extracted residues of carbonate stones which may, within limits, correspond to the sugar content we found in several calcified stones (unpublished). *Lee* et al. (13) did not use the native material for sugar analysis in gallstones (n = 10, cholesterol stones = 7, pigment stones = 3), but started from a precipitate called the "soluble glycoprotein". The dry weight of this fraction was 0.15% of the total weight, and they determined a sugar content of 55–75% by GLC, corresponding to 0.09% of the total weight. This agrees more closely with our findings than the figures of *Good* et al. (10) and *Tandon* et al. (29). Presuming a carbohydrate moiety of 70–80% for intestinal glycoprotein (27, 28) we calculated from the measured sugars an approximate amount of 0.12% of glycoprotein for the sugar containing cholesterol stones/mixed stones and 0.5% for pigment stones. Referring to these figures we suppose that the amount of glycoprotein in pigment stones and mixed stones has

been overestimated (10, 29) or underestimated by others (13). The former may be attributed to interferences in protein assay by bile pigments, leading to false-positive results, the latter to substance losses during extraction and to an inadequate GLC assay of sugars. i.e. using packed columns as reported by *Lee* et al. (13).

The presence of rhamnose in nearly all samples containing sugars, but above all in pigment stones, suggests that some part of the glycosubstances must be of bacterial origin, since rhamnose is a constituent of the o-antigen of gram-negative bacteria. These findings should be taken into consideration when discussing the role of bacterial infection in pigment stones pathogenesis. In fact, the largest portions of rhamnose have been found in pigment stones, and this result was rather significant when compared with the group of cholesterol stones and mixed stones (pigment stones/cholesterol stones and mixed stones: $p = <0.02$ and $p = <0.01$; cholesterol stones/mixed stones: $p = 0.15$). Admittedly, this cannot prove convincingly that pigment stones are caused by biliary infection, for the latter may be the result of the stones themselves. However, the latter argument should also be applied to cholesterol stones, for we certainly do not see why such concretions should lead to biliary infection less frequently than do pigment stones.

Significantly high proportions of rhamnose in pigment stones point to a bacterial history of this stone type, and we feel that this might be of a similar aetiological significance as is the finding of an increased bacterial β-glucuronidase activity in the bile of patients with pigment stones (30).

Acknowledgement

We thank Dr. *Fürst* from the Landesuntersuchungsamt Münster for performing the GLC/MS measurements.

Note added to proof

The investigations reported were based on stone material which was collected over a longer period of time and it was stored in a carefully dried and coolish state. However, recent investigations with a much smaller series of stones immediatedly analyzed after endoscopic extraction revealed much higher proportions of carbohydrates. From these studies results that proteins account for about 3% (pigment gallstones), 1.8% (mixed gallstones), and 0.5% (cholesterol gallstones). It may be suggested that some polymerization or degradation reactions take place during storage impairing methanolysis effectivity. The circumstances have to be clarified.

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